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13. ABSTRACT (Maximum 200 Words) Human mammary epithelial S1 cells cultured in a 3D reconstituted basement membrane (rBM) growth arrest and form polarized acini-like structures. This differentiation in 3D rBM is accompanied by rearrangements of the nuclear architecture. ECM itself influences gene expression and cellular function by diverse mechanisms including ligand-receptor interaction, promotion of cell-cell adhesion, and modulation of cell shape. The changes in gene expression that occur during differentiation or tumorigenesis are accompanied by characteristics patterns of chromatin reorganization, modulated, in part, through highly regulated, histone acetylation/deacetylation mechanisms. We found that the differentiation of S1 cells into acini is accompanied by deacetylation of histone H4. Here we tried to elucidate the mechanisms by which the cellular microenvironment signals to nuclear acetylation/deacetylation events in the S1 cells. The rBM induced histone 4 deacetylation is neither directly related to growth nor to the ECM-induced signaling. We assessed the role of cell shape on histone H4 acetylation. Cells cultured on the non-adhesive substratum polyHEMA round up and form multicellular aggregates. In this context, they also display a significant deacetylation of histone 4. Using cytochalasin D to disturb the actin filaments, we are now analysing the role of actin organization in this process.				
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INTRODUCTION

The Bissell laboratory used a three-dimensional culture assay in which mammary epithelial cells are grown within a reconstituted basement membrane (3D rBM) to characterize normal and malignant human breast cell behavior. When plated on standard tissue culture plastic (2D), both normal and tumorigenic human mammary epithelial cells (HMECs) exhibit a flattened morphology. When cultured in 3D rBM, however, the non-malignant cells arrest growth, undergo a process of morphogenesis to form a polarized acinar structure that secretes sialomucins apically and deposits basement membrane components basally (Petersen et al., 1992). More recently, the laboratory has used a human breast cancer progression model that employs serially propagated cells that share a common genetic background (for review see Bissell et al, 1999). These cell lines are referred to as HMT3522- S1 (non malignant) and T4-2 (malignant) (Briand et al, 1987). In 3D rBM, S1 cells form a polarized acinar structure, while T4-2 cells continue to proliferate; in this context (3D culture) the T4-2 cells can be reverted to the "normal" phenotype when treated with inhibitory antibodies or chemical inhibitors against EGFR or beta-1 integrins (Weaver et al., 1997; Wang et al., 1998).

Dissecting the signaling pathways lead to cancer requires a fundamental understanding of the mechanisms that govern tissue organization, cell growth and differentiation and cell response to their microenvironment in normal tissues. Normal cells determine to differentiate or to proliferate as an integrated response to cues derived from extracellular matrix (ECM) molecules, soluble factors, and cell-cell interactions (Gumbiner et al., 1996; Bissell et al, 1999). These responses produce particular patterns of gene expression that are cell and tissue specific. The changes in gene expression that occur during differentiation and tumorigenesis are accompanied by characteristic changes in chromatin organization (Davie et al., 1999), defined by the supramolecular arrangement of nuclear proteins into dynamic complexes. These proteins include histones, transcription factors, and the enzymes that alter their acetylation status (Ogryzko et al., 1996; Wolfe et al., 1998; Liu et al., 1999). Both histone acetylation and deacetylation appear to be important in differentiation and neoplastic processes, depending on the target gene involved (Archer et al., 1999), though it remains to be clarified how these events are coordinated to produce complex phenotypic changes.

The structural differentiation of the non-malignant human mammary epithelial cell line S1 cells in 3D rBM is accompanied by rearrangement of the nuclear architecture (Lelievre et al., 1998). This specific nuclear organization appears to be important for establishing and maintaining the normal cell phenotype, and by extension, the normal pattern of gene expression. It has been shown in many different cell models that changes in chromatin condensation (heterochromatization) accompanies cellular differentiation (Francastel et al., 2000), a process in which histones become deacetylated. The level of histone acetylation is determined by equilibrium between histone acetyltransferase and deacetylase (Wolfe et al., 1996). The observation that for chromatin structure can be precisely modulated, through highly regulated, reversible histone acetylation/deacetylation mechanism suggests that nuclear reorganization is an essential component of dynamic transcriptional silencing or activation. Understanding how signals from the microenvironment that act to induce differentiation control these histone acetylation/deacetylation mechanisms is the central goal of this proposal.

BODY

The initial proposal was based on 3 preliminaries results, some aspects of which proved difficult reproduced (part I). Some new experiments, not planned in the statement of work, have therefore been done to further our principal objective; they are described in the part II. Since the preliminary results were not fully reproduced and since new experiments were designed and new results were obtained, the research accomplishments are not completely associated with each task outlined in the approved Statement of Work. Nevertheless, I believe that the progress of our work and the research findings we have obtained since last summer, when this proposal was submitted, are of general interest and will result in a publication.

PART I: FORMATION OF A GROWTH ARRESTED 3D STRUCTURE, AND NOT FORMATION OF A FULLY POLARIZED 3D STRUCTURE, IS THE KEY SIGNAL FOR BY A DECREASE OF HISTONE H4 ACETYLATION

1- Differentiation into acini in laminin-rich rBM is associated with decrease in histone H4 acetylation.

Our preliminary result showed a decrease of acetylated histone H4 in S1 cells after 10 days of culture in 3D rBM (fig 1A). At that time S1 have formed a well polarized, acini-like structure as compared with cells after only 4 days of culture in 3D rBM (fig 1B). One question resulting from those experiments was: Is the decrease of the histone H4 acetylation related to cell growth arrest or to the development of fully polarized, acini like, 3d structure?

2-The level of differentiation into acini-like structures doesn't seem to play a role in the overall H4 acetylation levels.

The second preliminary result suggested a decrease in acetylated H4 levels in S1 cells cultured in a laminin rich 3D rBM matrix as compared with a 3D collagen-I matrix (fig 2A), suggesting that the decrease of histone H4 acetylation was due to laminin-rich BM signaling rather than to the formation of a 3D structure per se. Indeed, non-malignant HMECs cultured inside collagen gels growth arrest and form spherical structures, but the level of sphere organization is different from the 3D rBM cultures. Indeed, cultured inside collagen I, and fail to deposit an endogenous basement membrane (Petersen et al., 1992 and Howlett et al., 1995) (fig 2Ba). They also fail to properly organize NuMa nuclear localization (Lelievre et al., 1998).

To compare the level of histone acetylation between these two 3d structures, we had first to extract the cells from the gel. Because of the nature of the collagen I gel, it was impossible to extract the cells from this gel at 4°C with a solution of PBS/EDTA as was done to extract the cells from the EHS gels. Therefore, we used an enzymatic protocol (using collagenase) and performed the extraction at 37°C, as recommended (fig 2A). We also observed a difference in the level of cyclin D1 between cells cultured inside EHS and cells cultured inside collagen I, using these two different protocols to extract the cells from the gels.

Consequently, we decided to verify if the difference observed in the acetylated histone 4 levels between the two 3D culture conditions (EHS versus collagen I) could be a consequence of the different protocols used to extract the cells. So, we extracted the cells from both EHS and collagen I at 37°C using an enzymatic protocol (dispase for EHS and collagenase for collagen). Since culturing HMEC in collagen gel containing 10% of EHS allows the cells to form polarized, acini-like structures as well, we also compared the level of histone acetylation between 3D collagen I cultures containing (or not) 10% of EHS, using the collagenase at 37°C to liberate the

cells from the gel. From those experiments we didn't see any significant differences in the level of histone acetylation (fig 2Bb and 2Bc) and concluded that the level of differentiation into acini structures doesn't play a role in H4 acetylation levels.

3-The level of p300 doesn't seem to be modulated by the degree of 3d structure organization.

Preliminary microarray results have found that an mRNA related to CBP/p300, which encodes a protein that is known to have histone acetyltransferase activity, is decreased when S1 cells are cultured within rBM as compared to cells cultured on tissue culture plastic. Unfortunately this result wasn't confirmed by other microarray analyses performed in the laboratory by Aylin Rizki (postdoc fellow), nor by western blot. Furthermore, other results suggested that the level of p300 was decreased when the S1 cells have growth arrest rather than from morphogenesis.

Nevertheless, since we observed a decrease of histone acetylation during the 3D structure formation, we transfected the S1 cells with a vector expressing p300 (Miller et al. 2000) to assess the role of histone acetylation in this morphogenic process. We observed extensive cells death during the first hours of transfection, perhaps due to the effect of p300 to p53 (acetylation of p53 increases its activity) (Liu et al., 1999). We were, however, able to isolate clones that have yet to be analyzed for the presence of the transgene and the overexpression of p300 before performing the 3D assay with those cells.

Based upon those results, our immediate objectives changed. The comparison between 3D cultures inside EHS or inside Collagen I was done to assess the level of histone 4 acetylation between two growth arrested 3D cell structures: one being reminiscent of polarized acini (3D culture in EHS) and the other one corresponding of spheres not well polarized (3D culture in collagen I). Since we didn't see any differences, our next priority was to identify if the histone H4 deacetylation observed during the 3D rBM morphogenesis was or not related to the growth arrest. We wondered also if this histone deacetylation was caused by the 3D structure formation, independently of the final differentiation of the structure. Different experiments were performed that led to some new findings regarding our understanding of ECM or 3D-induced histone 4 deacetylation in non-malignant HMT3522-S1 cells.

PART II: A ROLE FOR THE CELL SHAPE IN HISTONE H4 DEACETYLATION IN NON MALIGNANT S1 CELLS

ECM-directed growth arrest is an early and critical step in mammary epithelial cell morphogenesis (Petersen et al. 1992). To distinguish between the effect of ECM-directed growth arrest and changes caused by the 3D structure formation, we compared the level of histone acetylation between growth arrested and proliferating S1 cells cultured in monolayers (fig 3). Growth arrest was induced by removing EGF from the media of growing S1 cells (fig 3A, 3B) and was accompanied by a decrease in the level of acetylated histone H4 (fig 3C). Meanwhile we observed that EGF starvation results also in a change in the S1 cell morphology (fig 3A). EGFR has been previously shown to perturb adherens junctions in epithelial cell lines (Hazan et al. 1998, Al moustafa et al. 1999). Cadherin-mediated cell-cell interactions are involved in organizing f-actin into the sub-cortical cytoskeleton in epithelial cells (Gumbiner 1996; Braga et

al., 1999). Strong adherens junction and subcortical organization of actin cytoskeleton defined the epithelial cell morphology (Piepenhagen et al., 1998; Adams et al., 1999 ; Braga et al., 1999; Braga V., 2000), and are involved in the epithelial cell shape. To confirm that the EGF starvation was responsible for the change in cell morphology, we examined the distribution of E-cadherin and the organization of the actin cytoskeleton in control cells and in EGF starved cells. All the images were taken with the same time of exposure (fig 4 Aa, Ab, Ba, Bb), allowing us to compare the intensity of the staining. The inserts (figures 4 Ac, Ad, Bc, Bd) show a higher magnification of part of fig 4Aa, Ab, Ba, Bb, and are presented with the same contrast to allow comparison of the localization (or organization) of E-cadherin and f-actin staining. We observed a higher E-cadherin staining at the cell-cell contact (fig 4Aa and Ab) and almost not fuzzy cytoplasmic staining of E-cadherin in EGF starved cells (fig 4Ac and Ad). The f-actin exhibit also a better organized and sharper cortical distribution in EGF starved cells (fig 4B).

Since histone hyperacetylation, rather than histone deacetylation, has been related to growth arrest in many cell types, we addressed the question of whether the histone acetylation levels control the growth status in S1 cells. We treated the S1 cells with the HDAC inhibitor sodium butyrate (NaBu) to produce a histone hyperacetylation, and observed its effect on growth and on cell morphology. We compared the effects of NaBu treatment to EGF starvation. In both cases the cells growth arrest, as measured by a decrease in the Ki-67 labeling index (fig 5A), but display different morphology (fig 5B). Indeed, EGF-starved cells form "smooth round" islands, although NaBu-treated cell morphology is not very different from control cells (fig 5B). As shown previously, EGF starvation leads to a decrease of histone acetylation (fig 5C, lane 2). As expected NaBu treatment increase drastically the level of histone H4 acetylation (figure 5C lane 3).

Thus, both EGF starvation and NaBu treatment led to cell growth arrest, but have different effect in histone acetylation/deacetylation process and on cell morphology. Dramatic histone hyperacetylation induced growth arrest in S1 cells cultured on tissue plastic. This suggests that the effect of EGF removal on histone deacetylation is not directly related to its effect on growth but could be caused by its effect on cell morphology.

S1 cells growth arrest and undergo morphogenesis to form polarized 3D structures when cultured in a 3D rBM. This morphological differentiation does not occur when S1 cells are cultured as 2D monolayers (fig 6A). S1 cells were cultured for 10 days on tissue culture plastic or in a 3d rBM. There was no difference in cell cycle between the different culture conditions as shown by FACS analysis (fig 6B). The level of acetylated histone H4 decreases in 3D cultures compared to 2d cultures (fig 6C). Therefore the overall histone H4 deacetylation is the consequence of the cell morphology change induced by the 3D culture and is not related to the growth status.

ECM influences gene expression and cellular function by diverse mechanisms including ligand-receptor interaction, promotion of cell-cell adhesion, and modulation of cell shape. Our results suggested that the ECM-induced histone H4 deacetylation in S1 cells could be related to a change in cell shape. To address this question, we cultured the S1 cells on the non-adhesive substratum (polyHEMA). Under these conditions, the cells remain in suspension and become rounded and aggregated (fig 7A). We measured the level of acetylated histone H4 in cells

cultured on polyHEMA and observed a significant decrease in the level of acetylated histone H4 without any variation in the level of total histone H4 (fig 7B).

This change in cell shape could very well be the result of the change in the F-actin organization and/or a concomitant reorganization of adherens junction (Piepenhagen and al. 1998; Braga et al, 1999). We are currently investigating the role that E-cadherin and cortical actin organization could play in this regulation.

Our primary objective was to identify the mechanism by which signals from the ECM control the histone acetylation status in S1 cells. ECM is known to induce cell shape change. Our results suggest that the histone H4 deacetylation observed in the 3D structures formed by S1 cells is related to a change in cell shape (and/or change in cell adhesion) occurring during the differentiation induced by the ECM.

REPORTABLE OUTCOMES

1-Key research

a-Decrease of acetylated histone H4 associated of 3D rBM-induced morphogenesis in the non-malignant mammary epithelial cell line HMT3522-S1.

b-The organization of the S1 cells in a 3D structure is accompanied by a decrease of histone H4 acetylation, but the level of sphere organization doesn't interfere with the level of histone acetylation.

c- The overall histone H4 deacetylation is the consequence of the cells morphology change induced by the 3D culture and is not related to the growth status

d-EGF starvation leads to histone H4 deacetylation and cell morphology change.

e-Chemical-induced histone hyperacetylation induced S1 cells to growth arrest but didn't affect the cell morphology.

f-A rounded cell shape as opposed to a flattened morphology induces a histone H4 deacetylation.

2- Employment or research opportunity

The present PI of this award is a candidate for an assistant professor position (permanent position) in the service of the Pr. J. Chambaz at the medical school Broussais-Hotel Dieu and at the Unit 505 INSERM in Paris (FRANCE). This position will involve:

- Clinical research to improve molecular diagnosis of cancers at the Hotel Dieu hospital.
- Teaching of cell biology to students at the medical school Broussais-Hotel Dieu.
- Development of academic research project based on the study on the relationship between cellular microenvironment, nuclear organization and gene expression in epithelial tissue.

This training and the results described in this report constitute part of the proposal submitted for this employment application.

3-manuscript

A paper is being prepared based on the results presented in this report. Additional experiments are in process to complete this manuscript for submission.

CONCLUSION

We have demonstrated that ECM induces a histone H4 deacetylation. This result is neither due to an effect of ECM on growth nor on polarity, but rather to an effect of ECM on cell shape and/or cell-cell interactions. Tissue structural organization is the first step toward the acquisition of its function. Cell shape is determined by the environment (ECM, others cells, growth signals) and depends on the organization of the cytoskeleton. Furthermore, cell morphology change has been shown to affect gene expression (Roskelley et al. 1994, Close et al., 1997). Here we show that cell shape change induces a global histone H4 deacetylation. This could be one of the first responses of the non-malignant cells to ECM, which could directly lead to changes in genes expression (either direct repression or indirect activation).

Since T4-2 cells don't respond properly to ECM (Weaver et al. 1997, Wang et al. 1998), it would be now interesting to analyze if T4-2 cells has kept the ability to change their histone 4 acetylation status when cultured in 3D rBM as compared to tissue culture plastic.

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APPENDICES: FIGURES LEGENDS AND FIGURES

Figure 1:

- A. Levels of acetylated histone H4 in S1 cells grown on top of rBM for 4 days and for 11 days. Cells grown for 4 days and for 11 days were isolated from the EHS gels with dispase, proteins were extracted and equal amount were used for western blotting analysis. The amount of acetylated histone H4 was determined and normalized to the band corresponding to total histone H4 visualized by ponceau red staining. The normalized acetylated H4 level at day 11 is expressed as relative units to the normalized acetylated H4 level at day 4.
n=5, * : $p < 0.05$
- B. Merge images of Immunostaining for alpha 6 integrin (2^{ndary} ab labeled with FITC), Beta-catenin (2^{ndary} ab labeled with Texas Red), and DNA staining (DAPI). Left, S1 cells grown on top of EHS for 4 days, note that the alpha 6 integrin is basolaterally localized. Right, S1 cells grown on top of EHS for 11 days, note that the alpha 6 integrin staining is now restricted to the basal side and the Beta-catenin staining is reinforced at the apical site.

Figure 2: Comparison of the level of acetylated histone H4 in S1 cultured for 10 days in 3D rBM, 3D collagen or 3D collagen containing 10% of EHS)

- A. Acetylated H4 levels in S1 cells grown in 3D rBM (or 3D EHS) or grown in 3D collagen I (coll I) for 10 days. S1 cells were extracted from the 3D EHS) gels at 4 degree C with PBS/EDTA and from the 3D collagen I gels at 37 degree with collagenase. The amount of acetylated H4 was determined by western blot. Beta catenin and ponceau staining are used as loading control for the total amount of proteins loaded and for the amount of total H4.
- B. a) Immunostaining of laminin V, a component of the endogenous basement membrane produced by mammary epithelial cells, (2^{ndary} ab labeled with FITC) and DNA staining (DAPI) in S1 cells cultured in 3D EHS, 3D collagen I, 3D collagen I containing 10% of EHS (coll. I+10% EHS). Note that the cells grown in presence of EHS deposit basically the laminin V, but that cells grown in presence of collagen I only failed to deposit laminin V. b) The level of histone H4 acetylation levels in S1 cells grown in 3D rBM (or 3D EHS) or grown in 3D collagen I (coll I) for 10 days was measured by western blot. For both culture conditions cells were extracted from the gel at 37 degrees C with dispase (EHS) or with collagenase (Coll. I). Densitometric analysis of three independent experiments is shown, no statistical differences have been observed. c) The level of histone H4 acetylation levels in S1 cells grown in 3D collagen I (coll I) or grown in 3D collagen containing 10% of EHS (or 3D coll. I+10%EHS) for 10 days was measured by western blot. For both culture conditions cells were extracted from the gel at 37 degrees C with with collagenase. Densitometric analysis of two independent experiments is shown, the amount of acetylated histone H4 was normalized to the band corresponding to total histone H4 visualized by ponceau red staining, no statistical differences have been observed.

Figure 3: EGF starvation induces growth arrest and histone H4 deacetylation in tissue culture plastic.

- A. Phase contrast microscopy pictures of proliferating S1 cells cultured for 5 days on plastic (left) and of growth arrested S1 cells grown with EGF for 3 days, and without EGF for the last 2 days of the culture (right). Note the size of the cells and the formation of round islands in EGF starved cells
- B. Ki67 labeling index of S1 cells grown for 5 days in presence of EGF or EGF starved for the last 2 days. The values represent the percentage of Ki67 positive cells (determined by immunostaining of Ki67). More than 300 cells were counted.
- C. The level of histone H4 acetylation levels in S1 cells grown for 5 days on plastic with EGF or without EGF for the last 2 days of the culture was measured by western blot. The amount of acetylated histone H4 was normalized to the band corresponding to total histone H4 visualized by ponceau red staining. The normalized acetylated H4 level in cells +EGF is expressed as relative units to the normalized acetylated H4 in EGF starved cells. N=10, *: $p < 0.05$. A representative western blot is shown.

Figure 4: EGF starvation induces a S1 cells morphological change.

- A. Immunostaining of E-cadherin (2ndary ab labeled with FITC) and DNA (DAPI) in S1 cells EGF starved (b and d) or not (a and c). The images a and b were taken with the same time of exposure. The images c and d are higher magnification of the inserts in a and b, with adjusted contrast to allow a better comparison of the protein localization.
- B. F-actin and DNA staining in S1 cells EGF starved (b and d) or not (a and c). The images a and b were taken with the same time of exposure. The images c and d are higher magnification of the inserts in a and b, without the DAPI staining and with adjusted contrast to allow a better comparison of the protein localization.

Figure 5: Induced histone hyperacetylation leads to growth arrest.

- A. Ki67 labeling index of S1 cells grown for 5 days on tissue culture plastic. Control cells were cultured with regular medium containing EGF, EGF starved cells were cultured without EGF for the last 2 days, NaBu treated cells were cultured in presence of NaBu 5mM for the last 24 hrs. The values represent the percentage of Ki67 positive cells (determined by immunostaining of Ki67). More than 300 cells were counted.
- B. Phase contrast microscopy pictures of proliferating S1 cells cultured for 5 days on plastic (left), of EGF starved cells S1 cells (center) and of NaBU treated S cells. Note the size of the cells and the formation of round islands in EGF starved cells
- C. The level of histone H4 acetylation levels in S1 cells grown for 5 days on regular medium, without EGF for the last two days, or NaBu treated for the last 24 hours were measured by western blot. A representative western blot is shown. Beta catenin and ponceau staining show the equal amount of total protein and total histone 4.

Figure 6: Growth arrested 3D structures display a global histone H4 deacetylation as compared to growth arrested monolayer cells.

- A. Phase contrast microscopy pictures of S1 cells grown on tissue culture plastic (left) or on 3D rBM (right) for 10 days.
- B. FACS analysis of S1 cells grown on tissue culture plastic (left) or on 3D rBM (right) for 10 days.
- C. Western Blot analysis of proteins extracted from S1 cells grown on tissue culture plastic (left) or on 3D rBM (right) for 10 days. On the left a representative western blot for Ac H4, total H4 and ponceau staining is shown. On the right, the densitometric analysis of 4 independent experiments. The amount of acetylated histone H4 was normalized to the band corresponding to total histone H4 visualized by ponceau red staining. The normalized acetylated H4 level in cells in 3D is expressed as relative units to the normalized acetylated H4 cells in 2d. *: $p < 0.05$

Figure 7: A change in cell shape and cell adhesion lead to histone H4 deacetylation.

- A. Phase contrast microscopy pictures of S1 cells grown on tissue culture plastic (left) or on the non-adhesive substratum polyHEMA (right) for 4 days. Note the round shape on the cells on polyHEMA and the formation of 3D aggregates.
- B. Western Blot analysis of proteins extracted from S1 cells grown on tissue culture plastic (left) or on polyHEMA (right) for 4 days. On the left a representative western blot for Ac H4, total H4 and ponceau staining is shown. On the right, the densitometric analysis of 3 independent experiments. The amount of acetylated histone H4 was normalized to the total histone H4. *: $p < 0.05$

Figure 1 : acini like formation in laminin-rich rBM is associated with Histone H4 deacetylation

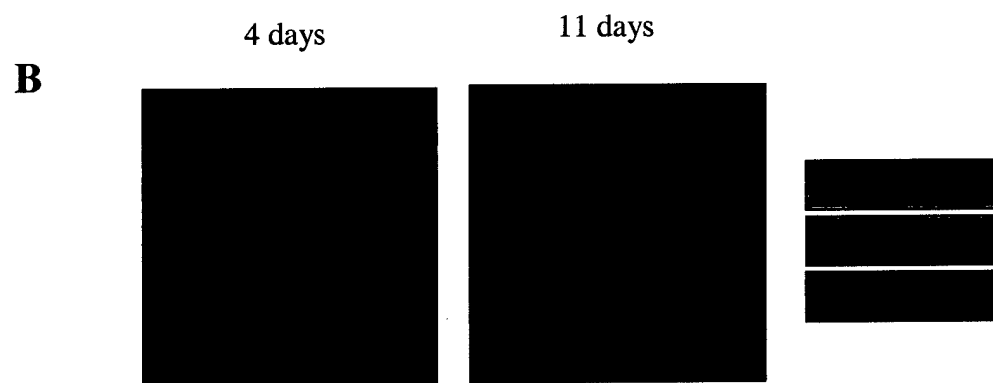
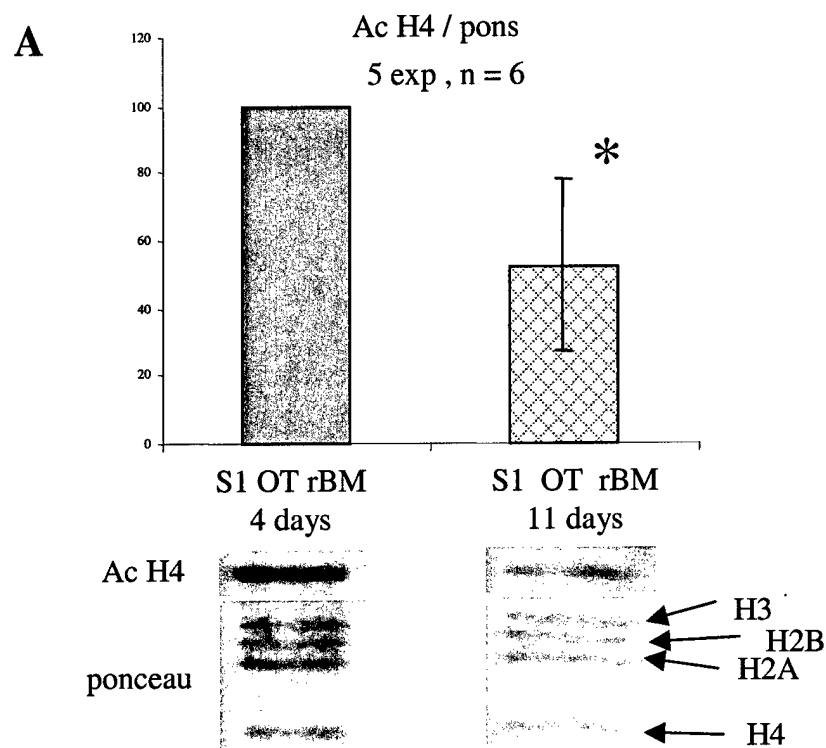
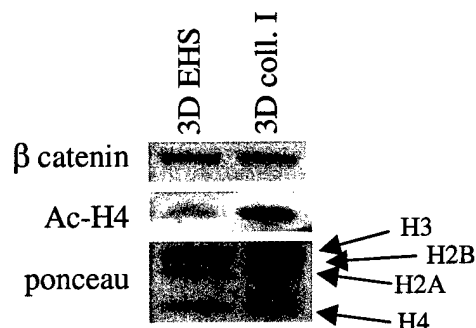


Figure 2: The level of differentiation of 3d structure doesn't play a role in the overall H4 acetylation

- A** Cell extraction protocol:
 3D EHS: PBS/EDTA at 4°C
 3D Collagen I: collagenase at 37°C



- B** Cells extraction protocol:
 3D EHS: dispase at 37°C
 3D Collagen I: collagenase at 37°C
 3D Collagen I+10%EHS: collagenase at 37°C

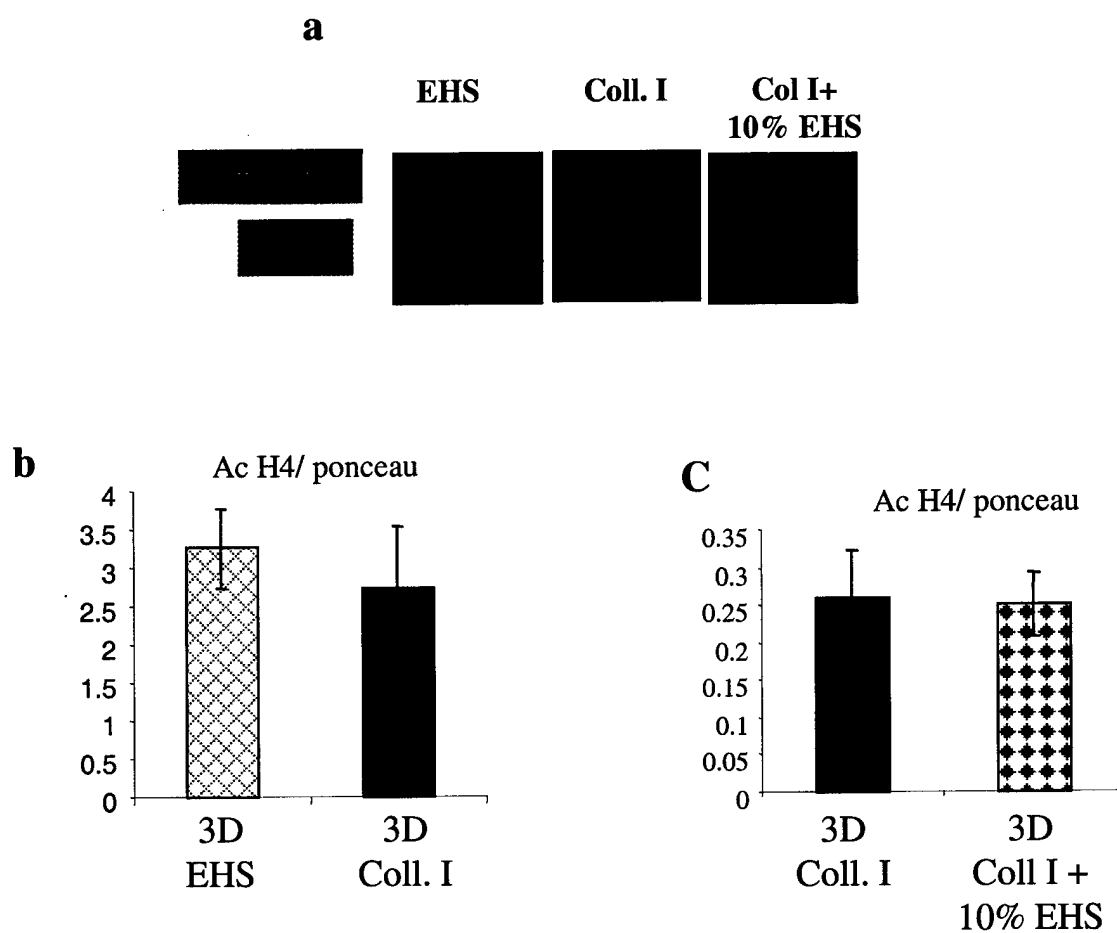


Figure 3: EGF starvation induces growth arrest and histone deacetylation in tissue culture plastic.

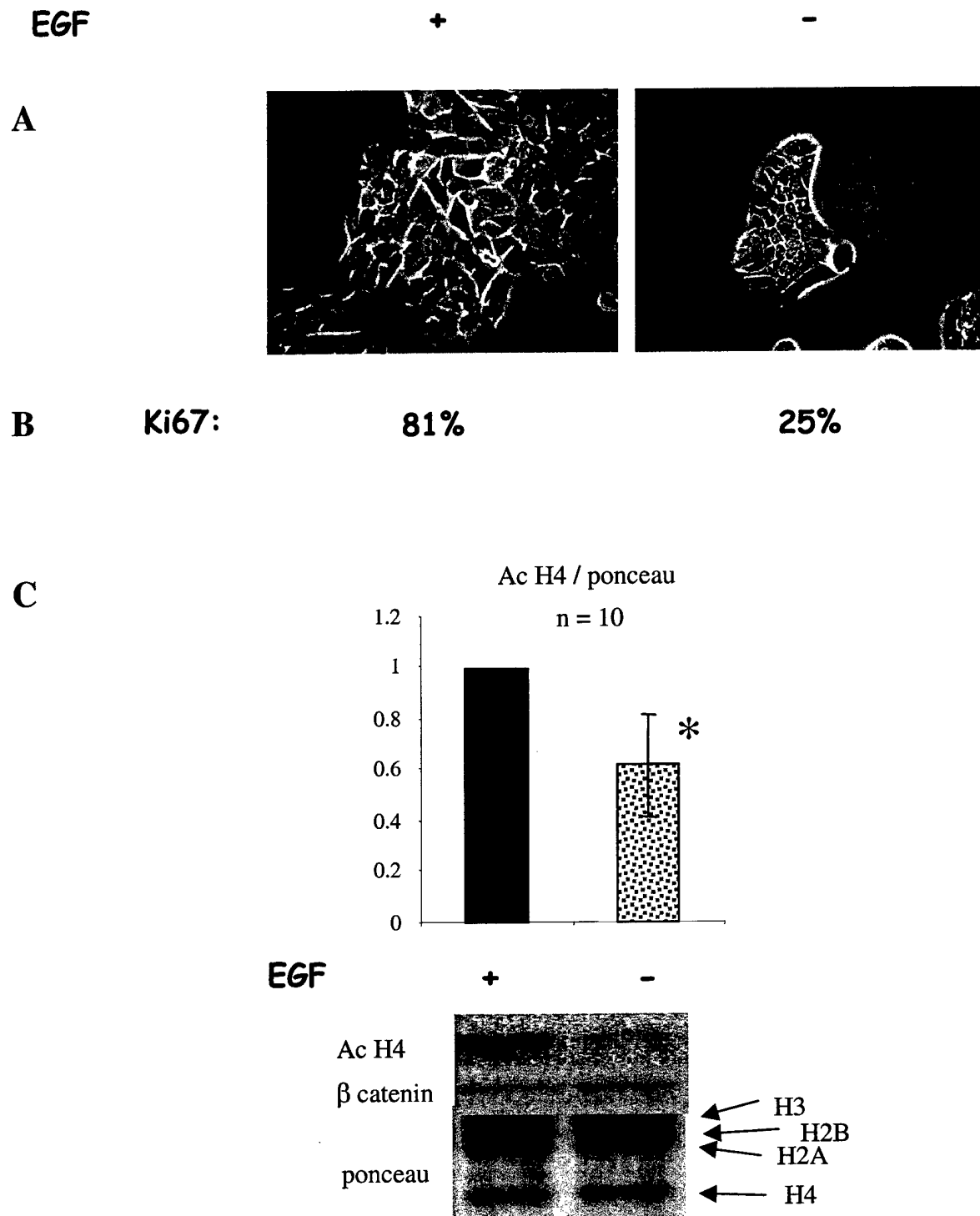


Figure 4 : EGF starvation induces a S1 cells morphological change

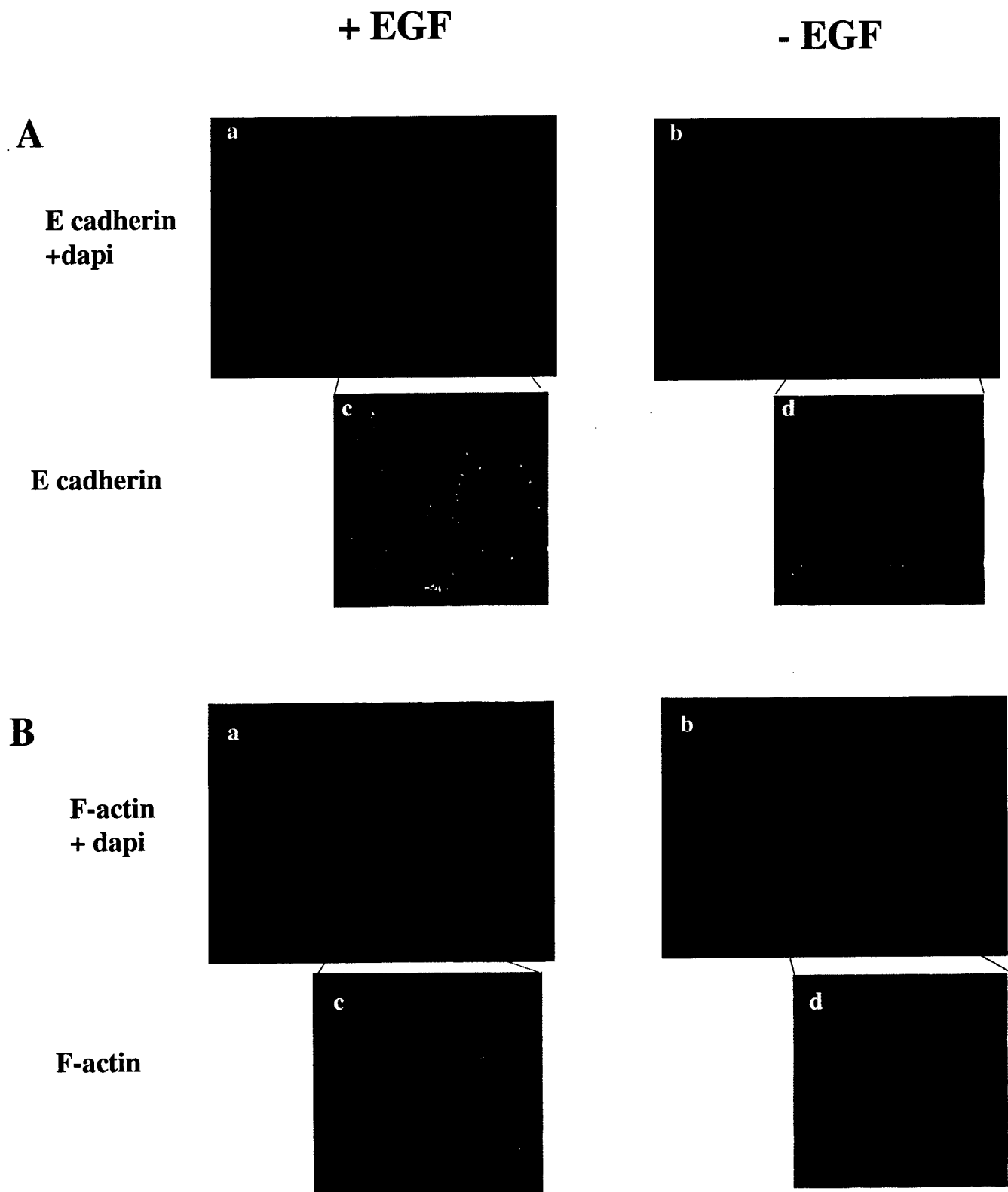


Figure 5: induced histone hyperacetylation leads to growth arrest

A

	Control cells	EGF starved cells	NaBU treated cells
Ki67	81 %	25 %	30 %

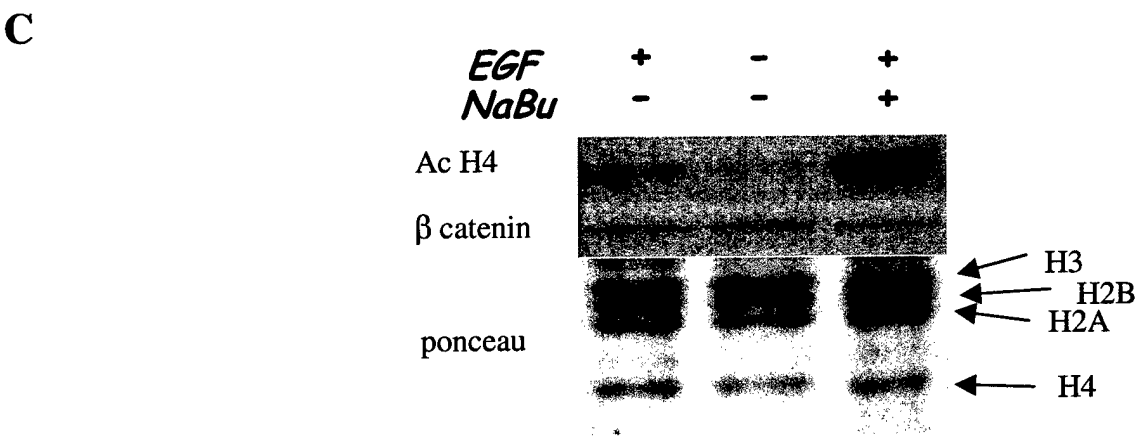
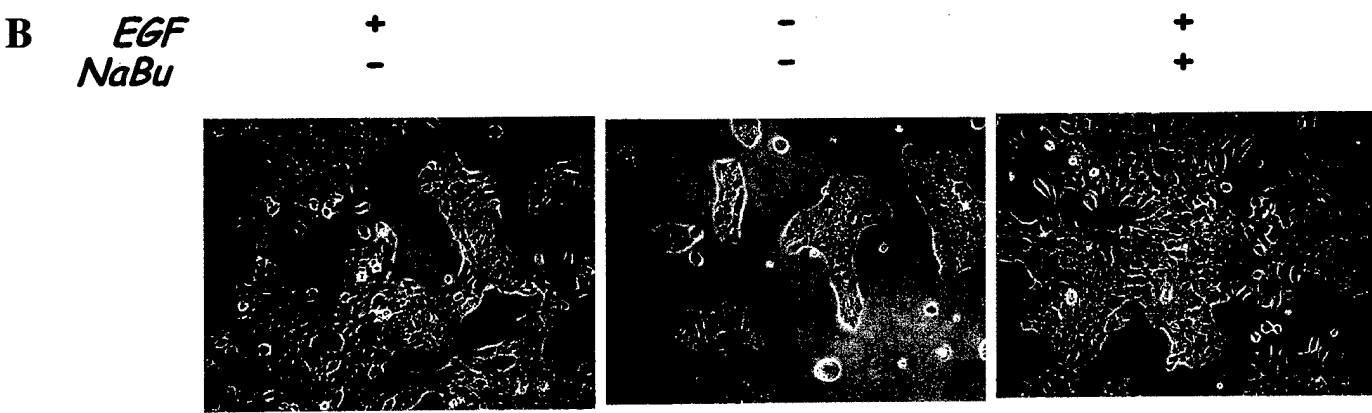


Figure 6 : growth arrested 3d structures display a global histone H4 deacetylation as compared to growth arrested monolayer cells.

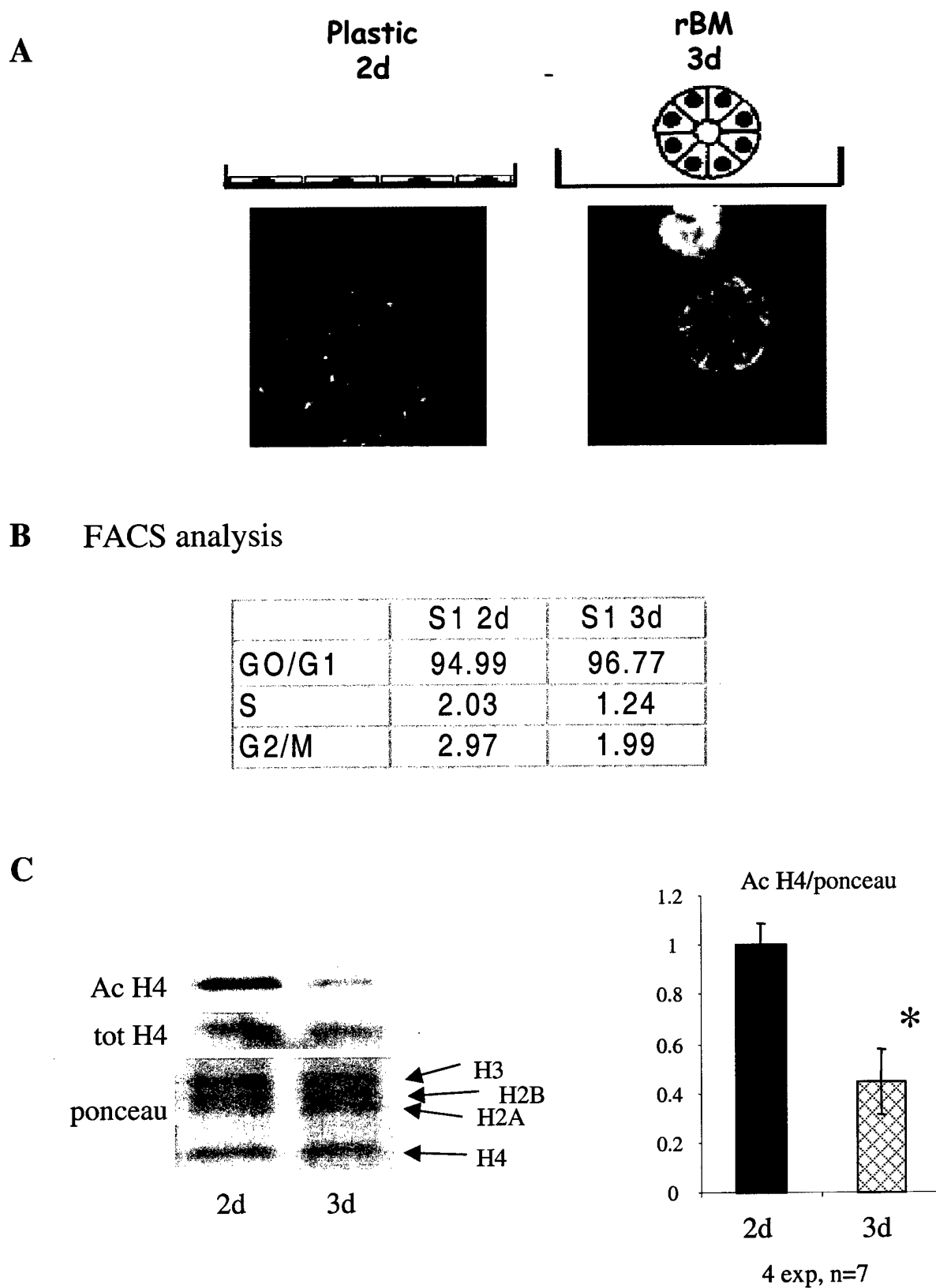


Figure 7 : A change in cell shape and in cell adhesion leads to H4 deacetylation

